

## EXAMPLE 16

### Effect Of Saliva Extract On The Invader-Directed Cleavage Assay

For a nucleic acid detection method to be useful in a medical (*i.e.*, a diagnostic) setting, it must not be inhibited by materials and contaminants likely to be found in a typical clinical specimen. To test the susceptibility of the invader-directed cleavage assay to various materials, including but not limited to nucleic acids, glycoproteins and carbohydrates, likely to be found in a clinical sample, a sample of human saliva was prepared in a manner consistent with practices in the clinical laboratory and the resulting saliva extract was added to the invader-directed cleavage assay. The effect of the saliva extract upon the inhibition of cleavage and upon the specificity of the cleavage reaction was examined.

One and one-half milliliters of human saliva were collected and extracted once with an equal volume of a mixture containing phenol:chloroform:isoamyl alcohol (25:24:1). The resulting mixture was centrifuged in a microcentrifuge to separate the aqueous and organic phases. The upper, aqueous phase was transferred to a fresh tube. One-tenth volumes of 3 M NaOAc were added and the contents of the tube were mixed. Two volumes of 100% ethyl alcohol were added to the mixture and the sample was mixed and incubated at room temperature for 15 minutes to allow a precipitate to form. The sample was centrifuged in a microcentrifuge at 13,000 rpm for 5 minutes and the supernatant was removed and discarded. A milky pellet was easily visible. The pellet was rinsed once with 70% ethanol, dried under vacuum and dissolved in 200  $\mu$ l of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA (this constitutes the saliva extract). Each  $\mu$ l of the saliva extract was equivalent to 7.5  $\mu$ l of saliva. Analysis of the saliva extract by scanning ultraviolet spectrophotometry showed a peak absorbance at about 260 nm and indicated the presence of approximately 45 ng of total nucleic acid per  $\mu$ l of extract.

The effect of the presence of saliva extract upon the following enzymes was examined: Cleavase® BN nuclease, Cleavase® A/G nuclease and three different lots of DNAPtaq: AmpliTaq® (Perkin Elmer; a recombinant form of DNAPtaq),

AmpliTaq® LD (Perkin-Elmer; a recombinant DNAPtaq preparation containing very low levels of DNA) and *Taq* DNA polymerase (Fischer). For each enzyme tested, an enzyme/probe mixture was made comprising the chosen amount of enzyme with 5 pmole of the probe oligonucleotide (SEQ ID NO:43) in 10 µl of 20 mM MOPS (pH 7.5) containing 0.1% each of Tween 20 and NP-40, 4 mM MnCl<sub>2</sub>, 100 mM KCl and 100 µg/ml BSA. The following amounts of enzyme were used: 25 ng of Cleavase® BN prepared as described in Example 9; 2 µl of Cleavase® A/G nuclease extract prepared as described in Example 2; 2.25 µl (11.25 polymerase units) the following DNA polymerases: AmpliTaq® DNA polymerase (Perkin Elmer); AmpliTaq® DNA polymerase LD (low DNA; from Perkin Elmer); *Taq* DNA polymerase (Fisher Scientific).

For each of the reactions shown in Figure 37, except for that shown in lane 1, the target DNA (50 fmoles of single-stranded M13mp19 DNA) was combined with 50 pmole of the invader oligonucleotide (SEQ ID NO:46) and 5 pmole of the probe oligonucleotide (SEQ ID NO:43); target DNA was omitted in reaction 1 (lane 1). Reactions 1, 3, 5, 7, 9 and 11 included 1.5 µl of saliva extract. These mixtures were brought to a volume of 5 µl with distilled water, overlaid with a drop of ChillOut® evaporation barrier (MJ Research) and brought to 95°C for 10 minutes. The cleavage reactions were then started by the addition of 5 µl of the desired enzyme/probe mixture; reactions 1, 4 and 5 received Cleavase® A/G nuclease. Reactions 2 and 3 received Cleavase® BN; reactions 6 and 7 received AmpliTaq®; reactions 8 and 9 received AmpliTaq® LD; and reactions 10 and 11 received *Taq* DNA Polymerase from Fisher Scientific.

The reactions were incubated at 63°C for 30 minutes and were stopped by the addition of 6 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 20% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. The products of the reactions were visualized by the use of an Hitachi FMBIO fluorescence imager, and the results are displayed in Figure 37.

0982667-101804

5 A pairwise comparison of the lanes shown in Figure 37 without and with the saliva extract, treated with each of the enzymes, shows that the saliva extract has different effects on each of the enzymes. While the Cleavase® BN nuclease and the AmpliTaq® are significantly inhibited from cleaving in these conditions, the Cleavase® A/G nuclease and AmpliTaq® LD display little difference in the yield of cleaved probe. The preparation of *Taq* DNA polymerase from Fisher Scientific shows an intermediate response, with a partial reduction in the yield of cleaved product. From the standpoint of polymerization, the three DNAPTaq variants should be equivalent; these should be the same protein with the same amount of synthetic activity. It is possible that the differences observed could be due to variations in the amount of nuclease activity present in each preparation caused by different handling during purification, or by different purification protocols. In any case, quality control assays designed to assess polymerization activity in commercial DNAP preparations would be unlikely to reveal variation in the amount of nuclease activity present. If preparations of DNAPTaq were screened for full 5' nuclease activity (*i.e.*, if the 5' nuclease activity was specifically quantitated), it is likely that the preparations would display sensitivities (to saliva extract) more in line with that observed using Cleavase® A/G nuclease, from which DNAPTaq differs by a very few amino acids.

20 It is worthy of note that even in the slowed reactions of Cleavase® BN and the DNAPTaq variants there is no noticeable increase in non-specific cleavage of the probe oligonucleotide due to inappropriate hybridization or saliva-borne nucleases.

### EXAMPLE 17

#### Comparison Of Additional 5' Nucleases In The Invader-Directed Cleavage Assay

25 A number of eubacterial Type A DNA polymerases (*i.e.*, Pol I type DNA polymerases) have been shown to function as structure specific endonucleases (Example 1 and Lyamichev *et al.*, *supra*). In this example, it was demonstrated that